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Cuticular hydrocarbons of the flea beetles, *Aphthona lacertosa* and *Aphthona nigriscutis*, biocontrol agents for leafy spurge (*Euphorbia esula*)

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Abstract

The adult beetles *Aphthona lacertosa* and *Aphthona nigriscutis*, used as biocontrol agents for leafy spurge, had a complex mixture of hydrocarbons on their cuticular surface consisting of alkanes, methylalkanes, alkenes and alkadienes as determined by gas chromatography-mass spectrometry. A trace amount of wax esters were present. In both species, the hydrocarbons were the major cuticular lipid class and the gas chromatographic profiles of the total hydrocarbons were similar. However, the profiles for the saturated hydrocarbon fraction were distinct for each species. Alkanes (*n*-alkanes and methyl-branched alkanes), alkenes and alkadienes comprised 26, 44 and 30%, respectively, for *A. lacertosa*, and 48, 26 and 26%, respectively, for *A. nigriscutis*, of the total hydrocarbons. The major methyl-branched hydrocarbons were 2-methylalkanes: 2-methyloctacosane and 2-methyltriacontane. The major monoene was hentriacontene and the major diene was tritriacontadiene. The species were unique in that a number of di- and trimethyl-branched alkanes were present in minor quantities in which the first methyl branch was on carbon 2 or 3. Examples of structures were 2,10-, 2,12-, 2,6-, 2,4- and 3,7-dimethylalkanes. 2,10,12-Trimethylalkanes and a 2,10,12,24-tetramethylalkane with one methylene between adjacent methyl branch points also were identified. The adjacent methyl branch points of the 2,4- and 2,10,12- and 2,10,12,24-methyl-branched alkanes appeared to cause additional fragmentations in the mass spectra. Dimethylalkanes with an odd number of carbons in the backbone of the molecule were identified as 2,23-dimethylnonacosane and 2,25-dimethylhentriacontane; their mass spectra also corresponded to mass spectra expected for a 2,6 branching sequence. However, a 2,6 branching sequence is not biosynthetically feasible because such a structure has a straight-chain tail with an odd number of carbon atoms beyond the last methyl branch point. The 2,23 and 2,25 branching sequences could be synthesized starting with a primer derived from the amino acid leucine which would account for both the even number of carbons between the branch points and an even number of carbons beyond the last methyl branch point.

Keywords: *Aphthona*; *lacertosa*; *nigriscutis*; Flea beetle; Hydrocarbons; Methylalkanes; Gas chromatography; Mass spectrometry; Leafy spurge; Cuticular lipids; Insects

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1. Introduction

Leafy spurge, *Euphorbia esula*, a native of Europe, was first reported in America in 1827 and has since become a major weed in rangeland and pastures in the northern United States and southwestern Canada (Dunn, 1985). The weed has an extensive and deep root system, and reproduces from seeds and vegetative stems from the roots, making it difficult to control (Lacey et al., 1984; Lavigne, 1984; Lym and Messersmith, 1987, 1994). Its milky latex is an irritant to cattle and horses but is tolerated by sheep and goats (Best et al., 1980; Kommendahl and Johnson, 1959). In Europe, natural enemies keep the weed under control (Gassmann and Schroeder, 1995). In 1986, introduced *Aphthona* flea beetle spp. were used in south central North Dakota to establish a field insectary which has provided millions of beetles for redistribution to other leafy spurge infestations in the north central Great Plains and upper Midwest. Although the flea beetles have established at numerous sites throughout this region, they have not established at all release sites. *Aphthona* flea beetles may be susceptible to environmental stressors such as drought, as well as extreme heat or cold, preventing them from establishing in habitats of adverse environmental conditions.

Cuticular hydrocarbons are chemically stable, major components of the surface lipids of many species of insects (Blomquist et al., 1987; Lockey, 1988, 1991; Nelson and Blomquist, 1995). Hydrocarbons may function to prevent desiccation, affect the adsorption of agricultural chemicals, provide a barrier to penetration by microorganisms, function as semiochemicals and serve as chemotaxonomic characters. The durability of hydrocarbons makes them a candidate for marking individual insects (Ginzel and Hanks, 2002). In order to more thoroughly characterize these flea beetles, we are reporting herein the results of the analysis of the hydrocarbon fraction of the cuticular waxes from two introduced species of *Aphthona* being used for biocontrol: *A. lacertosa* and *A. nigriscutis*.

2. Materials and methods

2.1. Insects

Aphthona flea beetle adults were collected from two sites near Valley City, North Dakota; one approximately 10 miles southeast and the other approximately 9 miles northwest of Valley City. A third sample was collected from a rangeland pasture in western North Dakota approximately 5 miles west of Medora. All collections were made on leafy spurge with a sweep net in the summer of 2000. The insects were immobilized in the cold and those for analysis were individually selected and separated according to species with the aid of a dissecting microscope. Identification keys for *Aphthona* flea beetles were used to identify *A. lacertosa* and *A. nigriscutis* (McDaniel et al., 1992; LeSage and Paquin, 1996).

2.2. Chemical analysis

Cuticular lipids were obtained by extraction of each sample (40–80 individuals) by slurrying in hexane for 1 min. Extracts were dried under nitrogen and resuspended in chloroform for analysis. The samples were analyzed for lipid classes by spotting an aliquot on thin-layer chromatography plates of 10×10 cm of HPTLC-GHL (Analabs®, Newark, DE) and developed in hexane/diethyl ether/formic acid (80:20:1, v/v/v). After drying, the developed plates were sprayed with 5% H_2SO_4 in 95% ethanol. After allowing the ethanol to evaporate the lipid bands were visualized by heating the plate in an oven at 160 °C for 10 min and then at 250 °C for 10–20 min. Band positions were compared with standards of tetratriacontane, arachidyl arachidate, hexacosanyl acetate, tristearin, heptadecanoic acid, 1-tricosanol, distearin, monostearin and phosphatidylcholine.

Monoene and diene hydrocarbons were obtained by chromatography on $AgNO_3$ silica gel (Alpha Products®, Danvers, MA) columns. The silica gel was heated at 160 °C for 60 min then allowed to cool slightly so that hexane could be added without flaming. A 0.5×9 cm column was prepared with the slurry of silica gel in hexane and washed with 40 ml hexane. A 200–500-μl aliquot of the sample in hexane was applied and fractions were collected in 5-ml portions as the eluting solvent was changed from hexane to benzene. The fractions contained: fraction 1—(hexane) saturated hydrocarbons; fraction 2 (25% benzene) monoenes; fraction 3 (50% benzene) dienes; fraction 4 (benzene) trace of dienes.

The samples were analyzed by gas chromatography-mass spectrometry (GC-MS) on a HP 5890A gas chromatograph equipped with a pressure programmable cool on-column injection port

and an autoinjector (Nelson et al., 2001). The column consisted of a 1-m retention gap connected to a 12.5-m×0.2-mm capillary column of cross-linked dimethylsilicone Ultra 1 (HP) and was coupled to a HP 5970B quadrupole mass selective detector. The carrier gas was He. The initial column temperature was set at 150 °C, then programmed to reach 320 °C at a rate of 4 °C/min, and held at 320 °C for 20–120 min as necessary for all components to elute. An aliquot of 1 μl in chloroform was injected (Figs. 1–4). Mass spectra for Fig. 5 were obtained on a HP 6890 gas chromatograph coupled to a HP 5973 quadrupole mass selective detector using a 40 m×0.18 mm i.d. DB-1 column and a 1-m retention gap connected to a cool on-column inlet with a constant He flow of 0.6 ml/min. Temperature was programmed from 150 to 220 or 240 °C at 20 °C/min, then at 3 °C/min to 270 or 290 °C, then at 4 °C/min to 325 °C and held for approximately 60 min. Mass spectra of the hydrocarbons were interpreted as previously described (Nelson, 1978; Blomquist et al., 1987; Nelson, 1993; Bernier et al., 1998; Carlson et al., 1998).

2.3. Lipid quantification

Total amount of cuticular lipid hydrocarbons, *n*-alkanes, methylalkanes, alkenes and alkadienes, were determined by GC-MS analysis. Total ion current data were analyzed using a computer spreadsheet in Lotus123™ in which the detector response was corrected for lack of linearity by using a standard curve described by three equations (Nelson et al., 2001). The data used to develop the equations were obtained by injection of a standard mixture of tricosanyl acetate, 3-methyltricosane, octacosane, tetracontane and tricosanyl heptadecanoate. The equations used for the ranges from 0 to 3 ng and from 100 to 1000 ng were linear, while the mid-range from 3 to 100 ng was best described by a 1st order polynomial equation. The equations describing the dose–response for each component of the standard mixture were calculated using Prism 2.0™ (GraphPad Software, San Diego).

3. Results

3.1. Thin-layer chromatography

The charred bands visible on TLC plates were similar for *A. lacertosa* and *A. nigriscutis* (data not shown). The dominant band was hydrocarbons which was shown to consist of the saturated, monoene and diene hydrocarbons by GC-MS. Faint bands were present corresponding to wax esters, free fatty acids, long-chain alcohols, mono- and diacylglycerols and at the origin where the sample was applied. Trace amounts of C40, C42, C44:1, C44 and C46 wax esters were confirmed by GC-MS. The major fatty acid moieties of the wax esters were C14, C16, C18:1, C18 and C20, respectively, for the five wax esters detected.

3.2. GC-MS of hydrocarbons

The total cuticular lipid samples were analyzed by GC-MS before separating the sample into alkanes, alkenes and alkadienes by $AgNO_3$ silica gel chromatography (Figs. 1 and 2). The overall profile for the total hydrocarbons was somewhat similar for the two species (Fig. 1a and Fig. 2a). Only hydrocarbons and wax esters were detected. Other compounds that would have been detected by GC-MS if present would have included: sterol esters; short-chain acid esters of long-chain alcohols; fatty acid esters; diacylglycerols; long-chain aldehydes; and if present in sufficient amounts, because some amount is 'lost' during chromatography, long-chain alcohols and free fatty acids.

The most abundant compound present in the major peak of each species was *n*-hentriacontene in *A. lacertosa* and 2-methyltriacontane in *A. nigriscutis*. However, the mixture of alkanes, methyl-branched alkanes, alkenes and alkadienes did not allow a clear determination of differences between the species and it was not possible to determine the amounts of each class of hydrocarbons. When the hydrocarbons were separated into alkanes (*n*-alkanes and methyl-branched alkanes), alkenes and alkadienes, differences between species and in their lipid classes became more apparent. *A. nigriscutis* had approximately 38% more total hydrocarbons than did *A. lacertosa*, 3900 vs. 2829 ng/insect, respectively (Table 1). *A. nigriscutis* had 2.5 times more alkanes than did *A. lacertosa* but had similar amounts (ng/insect) of alkenes and alkadienes.

It was apparent from analysis by GC-MS that the saturated hydrocarbons (Fig. 1b and Fig. 2b; Table 2) were largely 2-methyl-branched alkanes whereas the unsaturated hydrocarbons were a homologous series of monoenes (Fig. 1c and Fig. 2c) and dienes (Fig. 1d and Fig. 2d). The major

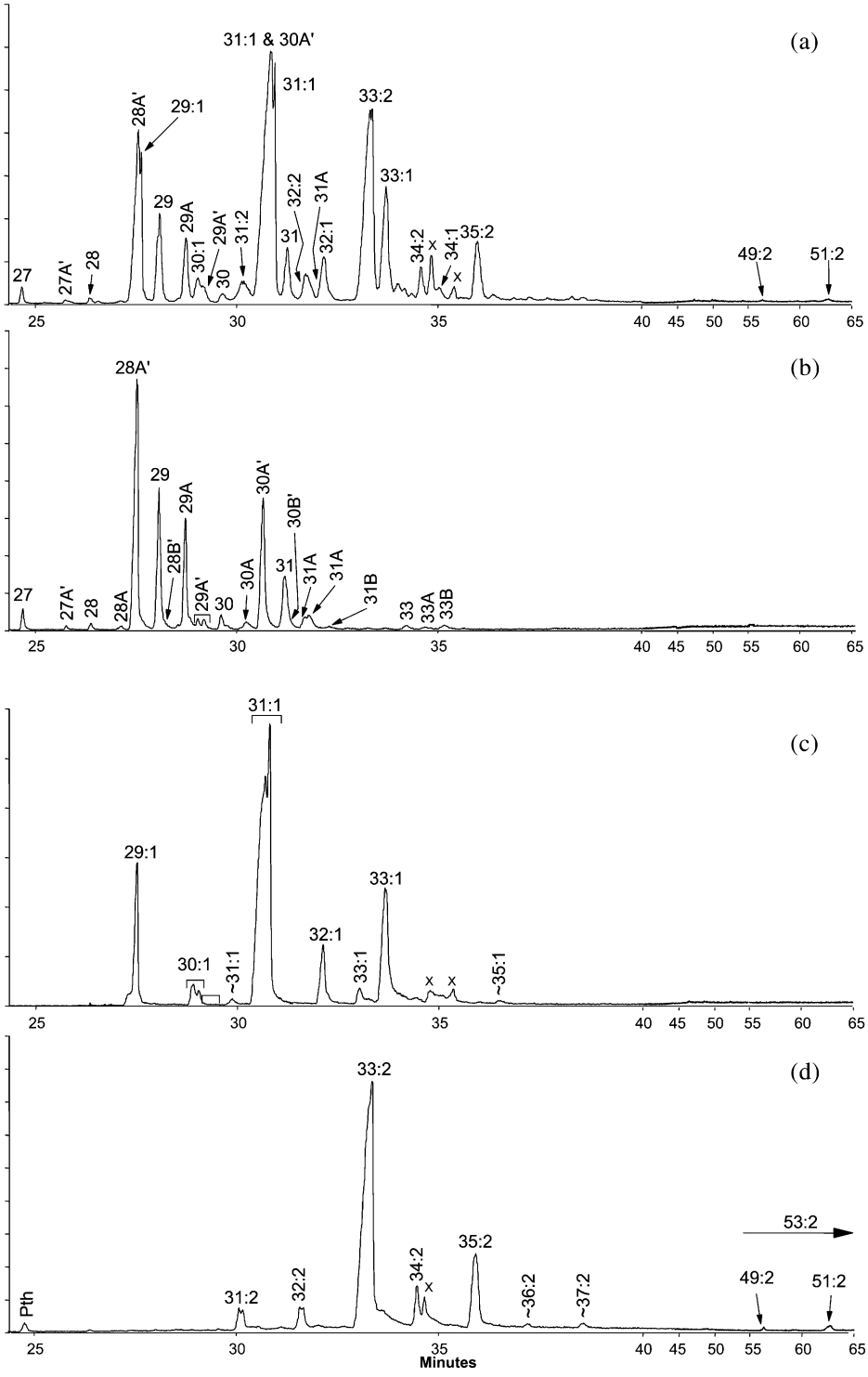

Fig. 1. GC-MS of cuticular lipids from adults of *A. lacertosa*: (a) total extract; (b) alkanes; (c) alkenes; (d) alkadienes. The number indicates the number of carbon atoms in the backbone of the molecule. The letters A, B and C indicate one, two, or three methyl branches, respectively. The :1 and :2 indicate one or two double bonds, respectively; '*x*' indicates a non-hydrocarbon, likely a contaminant. Although baseline peaks were present corresponding to the wax esters found in *A. nigriscutis* (Fig. 2a) their identity could not be verified.

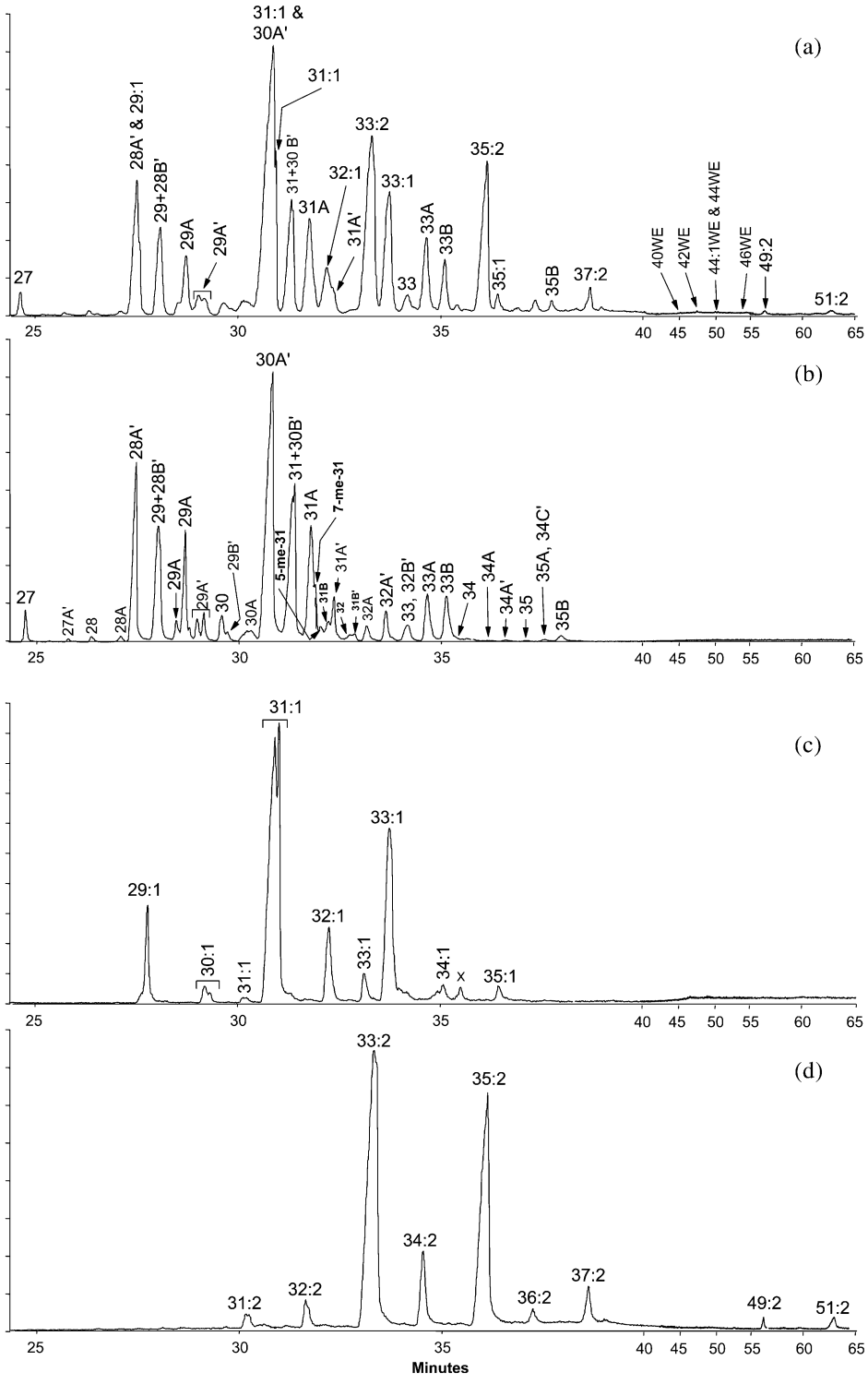

Fig. 2. GC-MS of cuticular lipids from adults of *A. nigriscutis*: (a) total extract; (b) alkanes; (c) alkenes; (d) alkadienes. The number indicates the number of carbon atoms in the backbone of the molecule. The letters A, B and C indicate one, two, or three methyl branches, respectively. The :1 and :2 indicate one or two double bonds, respectively. '*x*' indicates a non-hydrocarbon, likely a contaminant. 'WE' indicates wax esters.

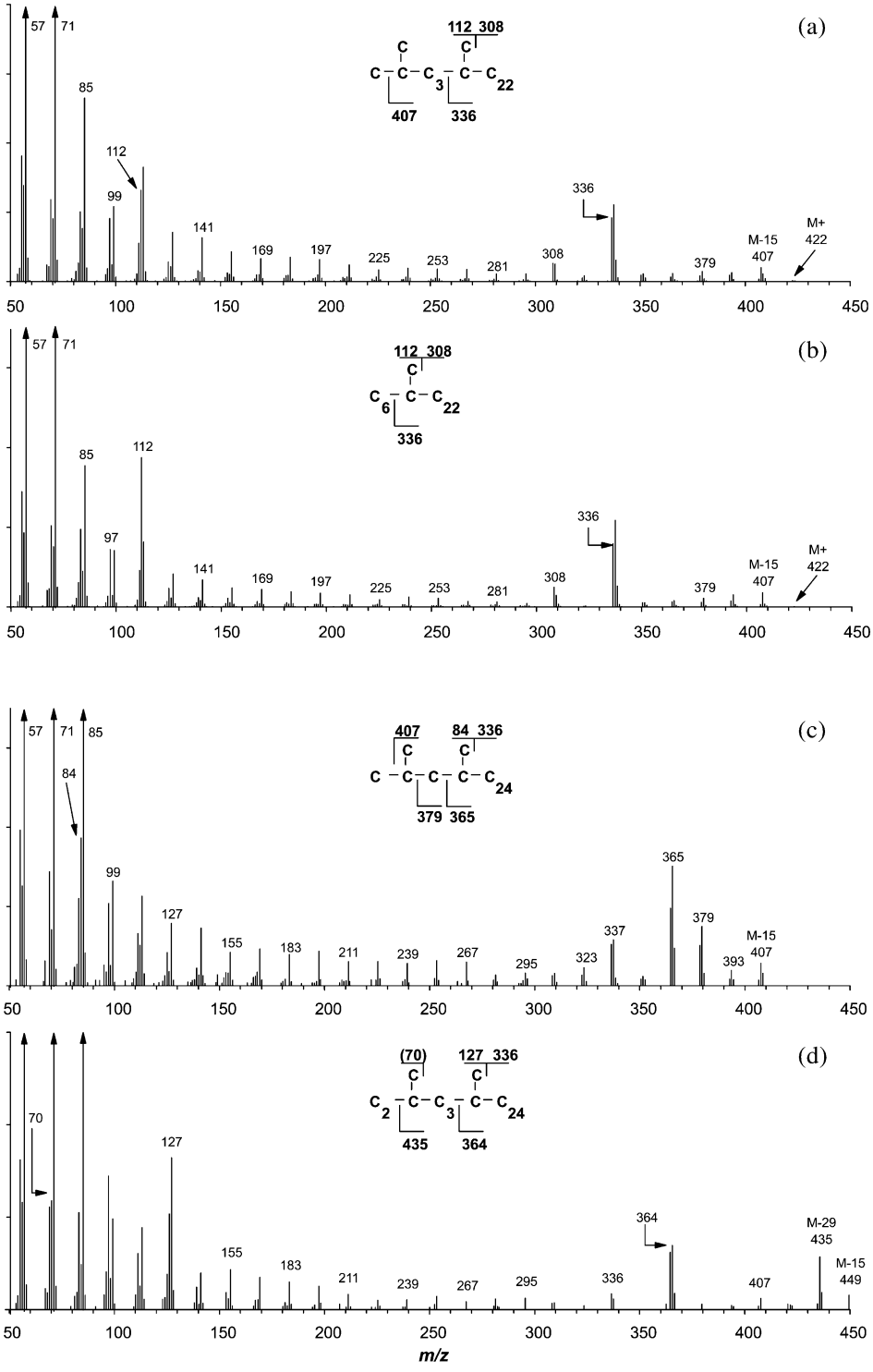

Fig. 3. Electron impact mass spectra of methyl-branched alkanes from *A. lacertosa*: (a) tailing edge of Peak 29, 2,6-dimethyloctacosane, (b) Peak 29A, 7-methylnonacosane, (c) tailing edge of Peak 29, 2,4-dimethyltriacontanes and from *A. nigriscutis*: (d) 3,7-dimethylhentriacontane. Where a pair of ions are of similar intensity, the most intense ion of the pair is labeled.

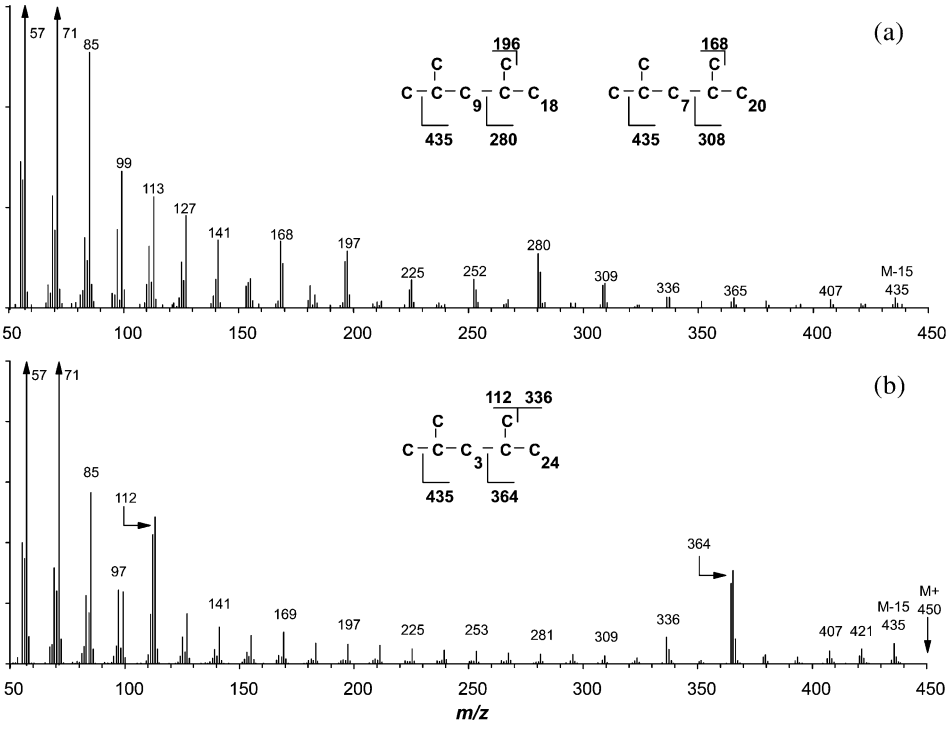

Fig. 4. Electron impact mass spectra of dimethyl-branched alkanes from *A. lacertosa*: (a) 2,12- and 2,10-dimethyltriacontanes; and (b) 2,6-dimethyltriacontane. Where a pair of ions are of similar intensity, the most intense ion of the pair is labeled.

alkane in *A. lacertosa* was 2-methyloctacosane (28.5%) and in *A. nigriscutis* was 2-methyltriacontane (24.3%) (Fig. 1b and Fig. 2b; Table 2). In fact, the entire GC-MS profile for the alkane fraction was shifted 2-carbons longer for *A. nigriscutis* compared to *A. lacertosa*. The monoene and diene profiles were not shifted between the species (Fig. 1c vs. Fig. 2c and Fig. 1d vs. Fig. 2d), however, the dienes were shifted 2 carbons longer than the monoenes in both species. The major monoene was hentriacontene (C31), 59.6 and 51.2% (Table 3), and the major diene was tritriacontadiens (C33), 46.6 and 67.8%, in *A. lacertosa* and *A. nigriscutis*, respectively (Table 4).

3.3. Mass spectra of B′ methylbranched alkanes

Dimethyl-branched alkanes in which one of the methyl branches is located on carbons 2, 3, or 4 of the carbon chain are designated as B′ alkanes. They elute near or with the *n*-alkanes one carbon shorter, e.g. 28B′ (30 carbons) elutes near or with nonacosane (29 carbons). Except for the 3,7-dimethylalkanes, which were at least partially resolved, these compounds were found 'hidden' in the leading and tailing portions of some *n*-alkanes (Fig. 1b and Fig. 2b; Table 2). The shortest chain length B′ compounds detected were 2,6- and 2,4-dimethyloctacosanes (28B′), which eluted on the tailing edge of peak 29 (nonacosane). The 2,6-isomer fragmented as depicted in Fig. 3a and could be mistaken for the internally branched monomethylalkane, 7-methylnonacosane (29A), except that the retention time was too short. The next peak to elute was the real 7-methylnonacosane (29A), which gave a similar mass spectrum (Fig. 3b). The major difference between the mass spectra was that the presence of the second methyl branch, on carbon 2, only partially suppressed the loss of a hydrogen atom from the 8-carbon dimethyl-branched fragment which resulted in the ion at m/z 113 being slightly greater than the ion at m/z 112 (Fig. 3a). The non-branched secondary fragment ion from the internally branched monomethylalkane gave the major ion at m/z 112 (Fig. 3b) as expected.

Also eluting on the tailing edge of peak 29 was the other 28B′, 2,4-dimethyloctacosane. This compound eluted immediately after the 2,6-isomer just before baseline was reached and was evident in

only three scans, as was the 2,6-isomer in the previous three scans, from an overloaded GC-MS analysis. The 2,4-isomer fragmented as depicted (Fig. 3c); the source of the ion at m/z 379 is not known although it could come from cleavage internal to the first branch point forming a branched 27-carbon fragment ion. A similar fragmentation has been observed for 13,15-dimethylheptacosane (Nelson et al., 1980), 13,15,17-trimethylnonacosane (Haverty et al., 1996), 2,18,20-trimethyltetratriacontane and 2,18,20-trimethylhexatriacontane (Nelson, 2001), and 13,15,19-trimethylhentriacontane and 13,15,21-trimethyltritriacontane (Nelson et al., 2001).

Peaks 30 (triacontane) and 32 (dotriacontane) were followed by 3,7-dimethylnonacosane and 3,7-dimethylhentriacontane, respectively. These B′ components were at least partially resolved from the *n*-alkanes. The 3,7-isomers fragmented similarly to that described for the 2,6- and 2,4-isomers, and in addition, had an increase in the intensity of the ion at m/z 70 (Fig. 3d). This ion cannot have originated by a simple carbon–carbon bond cleavage adjacent to a methyl branch point. It may have been formed by some type of rearrangement which resulted in a 5-carbon fragment of even mass, analogous to a McLafferty rearrangement, and formed an ion 15 atomic mass units greater than expected. However, the ion at m/z 70 was not a reliable indicator of 3,7-dimethylalkanes because the intensity was not consistently increased in all

Table 1
Nanograms per insect and percentage composition of the cuticular hydrocarbon classes[a]

Hydrocarbons	*A. lacertosa*		*A. nigriscutis*	
	Average	Percent	Average	Percent
Alkanes	746±62	26	1866±224	48
Alkenes	1231±234	44	1025±71	26
Alkadienes	851±165	30	1009±162	26
Total	2829±381		3900±382	

[a] Values are the average±standard deviation and the percentage composition of the hydrocarbon classes. Values are based on the analysis of seven aliquots of *A. lacertosa* and four of *A. nigriscutis* collected together by sweep net in North Dakota.

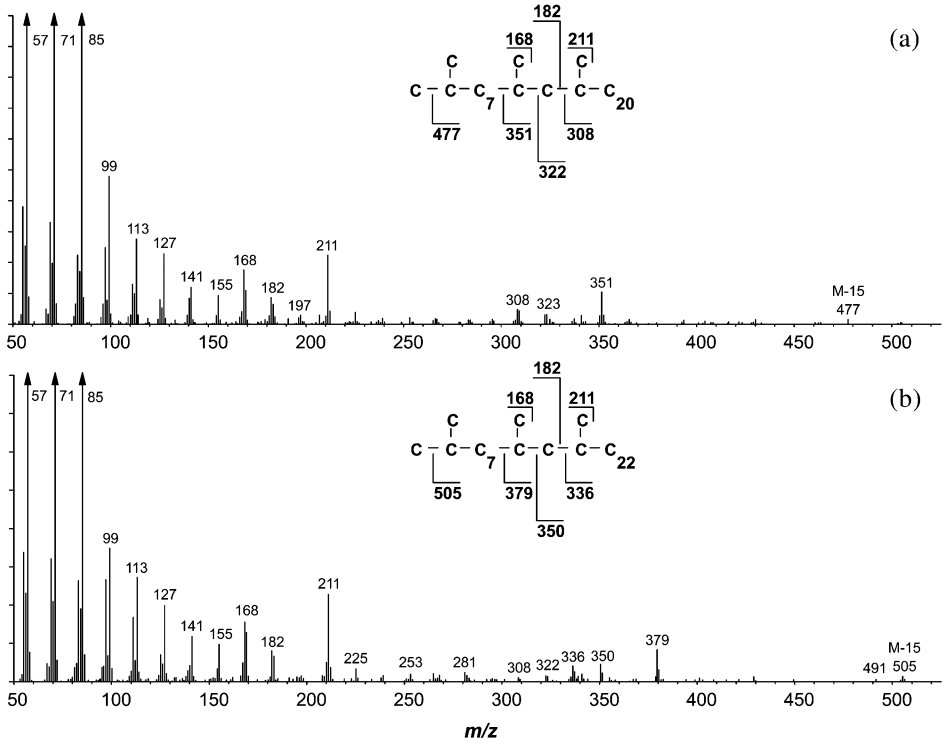

Fig. 5. Electron impact mass spectra of trimethyl-branched alkanes from *A. nigriscutis*: (a) 2,10,12-trimethyldotriacontane; and (b) 2,10,12-trimethyltetratriacontane. Where a pair of ions are of similar intensity, the most intense ion of the pair is labeled. Spectra were background subtracted.

Table 2
Percent composition of the *n*-alkanes and methyl-branched alkanes in the adult cuticular lipids[a]

GC-MS peak no.[b]	*A. lacertosa* average ± S.D.	*A. nigriscutis* average ± S.D.	Hydrocarbon[c]
21	t	t	Henicosane
22	0.0 ± 0.0	t	Docosane
23	0.3 ± 0.1	t	Tricosane
24	0.2 ± 0.1	t	Tetracosane
25	0.9 ± 0.2	0.5 ± 0.1	Pentacosane
26	0.3 ± 0.0	0.2	Hexacosane
26A′	0.2 ± 0.1	0.1	2-Methylhexacosane
27	2.2 ± 0.2	1.4	Heptacosane
27A′	0.5 ± 0.1	0.2	2*- and 3-Methylheptacosanes
28	1.0 ± 0.3	0.5 ± 0.1	Octacosane
28A	0.7 ± 0.1	0.5	7- and 6*-Methyloctacosanes
28A′	28.5 ± 1.3	11.5 ± 0.4	2-Methyloctacosane
29*,28B′	14.6 ± 0.7	9.0 ± 0.4	Nonacosane* and 2,6- and 2,4-dimethyloctacosanes
29A	0.6 ± 0.1	–	15-, 13- and 11-Methylnonacosanes
29A	–	1.0	13-Methylnonacosane
29A	10.7 ± 0.7	5.4 ± 0.5	7*- and 5-Methylnonacosanes
29A′	1.7 ± 0.2	1.0	2-Methylnonacosane
29A′	1.8 ± 0.2	1.3	3-Methylnonacosane
30	2.0 ± 0.2	1.3 ± 0.1	Triacontane
29B′	0.7 ± 0.2	0.7	2,23- and 3,7*-Dimethylnonacosanes
30A	1.9 ± 0.2	1.4 ± 0.2	14-, 12-, 10-, 8*- and 6*-Methyltriacontanes
30A′,29D′[d]	15.4 ± 0.7	24.3 ± 1.1	2-Methyltriacontane and ??^{T}
30B′,31*,30B′	8.1 ± 0.3	13.4 ± 0.3	2,12- and 2,10-Dimethyltriacontanes, hentriacontane* and 2,6-dimethyltriacontane
31A	3.5 ± 0.4	9.0 ± 1.6	15-, 13^{N}-, 11^{N}-, 9- and 7^{L}-Methylhentriacontanes
31A	t	0.8 ± 0.2	5-Methylhentriacontane
31A′,B	0.9 ± 0.3	1.0 ± 0.2	2-Methyl-^{T} and 11,15- and 11,21*-dimethylhentriacontanes
31A′,B	t	1.7 ± 0.5	3-Methyl*- and 5,9-dimethyl-hentriacontanes
32	0.4 ± 0.1	0.7 ± 0.6	Dotriacontane
31B′[e]	0.1	0.7 ± 0.2	2,25-, 3,11- and 3,7*-Dimethylhentriacontanes
32A,31C′	t	1.1 ± 0.1	12-, 10^{N}- and 8^{L}-Methyldotriacontanes* and 2,7,11-trimethylhentriacontane
32A′	t	1.5	2-Methyldotriacontane
32B	t	0.5 ± 0.5	6,10-Dimethyldotriacontane
32B′,33,32B′	1.0 ± 0.3	1.4	2,12- and 2,10-Dimethyldotriacontanes, tritriacontane* and 2,6-dimethyldotriacontane
33A	0.6 ± 0.2	–	13-, 11-, 9- and 7-Methyltritriacontanes
33A,32C′	–	2.9 ± 0.4	13- and 11-Methyltritriacontanes* and 2,10,12-trimethyldotriacontane
33B	1.4 ± 0.3	3.2 ± 0.5	11,21*-, 11,23- and 7,11-Dimethyl- and 3-methyl^{T} and 5,23-dimethyl^{T} tritriacontanes
33C	t	0.4 ± 0.2	7,11,21-Trimethyltritriacontane
34A	t	0.1	12-* and 10-Methyltetratriacontanes
34A′	t	0.2	2-Methyltetratriacontane
35	t	0.1	Pentatriacontane
35A	t	–	13- and 11-Methylpentatriacontanes
35A,34C′	?	0.3 ± 0.1	13- and 11-Methylpentatriacontanes* and 2,10,12-Trimethyltetratriacontane
35B,34D′[f]	t	0.7	11,23-Dimethylpentatriacontane* and 2,10,12,24-tetramethyltetratriacontane
36	t	t	?
37	t	t	?
37B	–	t	11,??^{N}-

Table 2 *(Continued)*

GC-MS peak no.[b]	*A. lacertosa* average ± S.D.	*A. nigriscutis* average ± S.D.	Hydrocarbon[c]
38	–	t	?
39	–	t	?
39B	–	t	11,??^{N}-

[a] Values are the averages and standard deviations of four samples of *A. lacertosa* and seven samples of *A. nigriscutis* determined from the total ion current plots from GC-MS. A 't' means the peak was present at less that 0.05%. Where no standard deviation is listed, the value was less than 0.05%.

[b] The GC-MS peaks correspond to those marked in Figs. 1 and 2. The number is the number of carbon atoms in the backbone of the molecule. The letters A, B, C and D indicate one, two, three and four methyl branches, respectively. A letter with a prime symbol means that one of the methyl branches is near the end of the molecule, i.e. on carbon 2, 3, or 4. Therefore, it is possible to have two or three peaks in sequence marked with a prime symbol. Where a peak is multi-component, the components are listed in their order of elution as determined by examining individual scans throughout the peak. If the major component could be estimated, it is marked with an asterisk. In two instances, peaks 29A and 33A, the components were so markedly different between the species that the data was entered separately for each.

[c] The individual hydrocarbons were determined from their electron impact mass spectra and their estimated equivalent chain lengths. If the major isomer could be determined, it was marked with an asterisk. If a peak component was present in a trace amount it is indicated with a superscript 'T'. If the major isomer was different between the species, the major isomer in each species is marked with a L (=*lacertosa*) or a N (=*nigriscutis*).

[d] 30A′ was such a large peak that it covered any B components that might have been present and resulted in the putative 29D′ eluting at the end of its tailing shoulder.

[e] The 2,25- and 3,11-dimethyl components were not detected in *A. lacertosa*.

[f] This component, 34D′, eluted on the tailing shoulder of 35B and the mass spectra supported a structure one carbon larger than 35B. The mass spectra had the same ions at m/z 168, 182 and 211 as were present in the mass spectra of 34C′. The other diagnostic ions were at m/z 350, 364 and 393, all one carbon greater than observed for 34C′.

Table 3
Percent composition of alkenes in the cuticular lipids of *Aphthona* adults[a]

GC-MS peak no.[b]	*A. lacertosa* average ± S.D.	*A. nigriscutis* average ± S.D.
23:1	0.0 ± 0.1	0.0 ± 0.1
25:1	0.3 ± 0.1	0.1 ± 0.1
27:1	0.3 ± 0.1	0.1 ± 0.1
28:1	0.1 ± 0.1	0.1 ± 0.1
29:1	11.0 ± 0.5	7.0 ± 0.2
30:1	2.9 ± 0.2	2.4 ± 0.0
31:1a	1.1 ± 0.2	1.4 ± 0.2
31:1b	59.6 ± 1.2	51.2 ± 3.7
32:1	5.8 ± 0.3	7.1 ± 0.6
33:1a	1.9 ± 0.4	3.2 ± 0.7
33:1b	16.6 ± 0.9	22.9 ± 2.1
34:1	0.3 ± 0.4	2.2 ± 0.3
35:1a	0.0 ± 0.0	0.3 ± 0.5
35:1b	0.2 ± 0.4	2.0 ± 0.2

[a] Values are the averages and standard deviations of four samples of *A. lacertosa* and seven samples of *A. nigriscutis* determined from the total ion current plots from GC-MS.

[b] Double bond positions were not determined. Three alkenes had isomers that eluted earlier than the main isomer(s). The first to elute was designated as 'a' and the later eluting peak as 'b'.

Table 4
Percent composition of dienes in the cuticular lipids of *Aphthona* adults[a]

GC-MS Peak No.[b]	*A. lacertosa* average ± S.D.	*A. nigriscutis* average ± S.D.
28:2	0.0 ± 0.0	0.0 ± 0.0
29:2	0.2 ± 0.3	0.0 ± 0.0
31:2a	3.1 ± 1.5	1.8 ± 0.4
31:2b	1.0 ± 1.2	0.2 ± 0.4
32:2	4.3 ± 0.4	2.8 ± 0.5
33:2	67.7 ± 1.7	46.6 ± 3.5
34:2	5.4 ± 0.8	8.0 ± 1.8
35:2	13.1 ± 0.6	31.1 ± 1.1
36:2	0.9 ± 0.1	1.3 ± 0.2
37:2	0.9 ± 0.1	2.4 ± 0.4
47:2	0.0 ± 0.0	0.1 ± 0.1
49:2	1.1 ± 0.1	2.0 ± 0.6
51:2	2.4 ± 0.5	3.7 ± 1.3

[a] Values are the averages and standard deviations of four samples of *A. lacertosa* and seven samples of *A. nigriscutis* determined from the total ion current plots from GC-MS.

[b] Double bond positions were not determined. One alkadiene had isomer(s) that eluted earlier than the main isomer(s). The first to elute was designated as 'a' and the later eluting peak as 'b'.

mass spectra, especially when resolution from other components was not complete.

Hentriacontane (peak 31) and tritriacontane (peak 33) contained 30B′ and 32B′ dimethylalkanes, respectively, eluting on both the leading and tailing edges of the peaks (Fig. 1b and Fig. 2b). The B′ alkanes were estimated to be the major components of peak 31. The leading edge of peak 31 had a mixture of 32-carbon compounds, 2,12- and 2,10-dimethyltriacontanes (the relative intensity of the ions at m/z 225 and 252 indicates a 2,14-isomer may also be present) (Fig. 4a). The mass spectra could also be interpreted as a mixture of the 32-carbon internally branched monomethyl alkanes, 13- and 11-methylhentriacontanes, except that the compounds eluted too early. On the tailing edge of peak 31, the third 30B′ component eluted, i.e. 2,6-dimethyltriacontane (Fig. 4b). The 2,6-isomer was estimated to be present in greater amounts than were the total of the 2,12- and 2,10-isomers. The next occurrence of similar isomers was in peak 33. However, in this case the *n*-alkane, tritriacontane, was the major component while the total of the 2,12- and 2,10-isomers appeared to be present in amounts comparable to that of the 2,6-isomer. A 2,4-isomer was not detected in either peak 31 or 33.

3.4. Mass spectra of C′ methyl-branched alkanes

Methyl-branched alkanes were detected on the tailing edge of peaks 33A in both species and on 35A in *A. nigriscutis* (Fig. 2b) indicating marked differences in structure than the earlier eluting components of the peaks. The elution positions indicated that the components may be monomethylalkanes in which the methyl branch position is shifted several carbons closer to the end of the chain, e.g. a 5- or 7-methylalkane vs. an 11- or 13-methylalkane, or a trimethyl-branched alkane in which one of the methyl branches was near the end of the carbon chain. The mass spectra on the tailing edge of peak 33A from *A. lacertosa* showed that it was a monomethylalkane, 7-methyltritriacontane.

However, the mass spectra from the tailing edge of peaks 33A and 35A from *A. nigriscutis* indicated that these components were 32C′ and 34C′, respectively (Fig. 5). The structures were interpreted from the mass spectra as being those of 2,10,12-trimethyldotriacontane (Fig. 5a) and 2,10,12-trimethyltetratriacontane (Fig. 5b). These structures are unique in that they have seven methylenes separating the first two branch points and only one methylene separating the second and third branch points. The mass spectra indicate that these structures have two additional fragmentations accompanied by loss of a hydrogen from the fragment ion: carbon–carbon bond cleavage on either side of the single methylene separating the two methyl branch points.

4. Discussion

The *Aphthona* species can be difficult to distinguish under field conditions (LeSage and Paquin, 1996). Size is not a good morphological characteristic because of size overlap among the species. Host plant also is not a good identification tool because all *Aphthona* species are host-specific to leafy spurge. Hydrocarbon profiles of insects and/or structures of methyl-branched alkane components have been used to differentiate closely related species (Lockey, 1988, 1991; Nelson and Blomquist, 1995). Although the collected adult *A. lacertosa* and *A. nigriscutis* were morphologically distinct, they had similar profiles and compositions of hydrocarbons. The greatest difference in their cuticular surface chemistry was in the profile of the saturated hydrocarbon fraction. Both species had a complex mixture of hydrocarbons requiring that the gas chromatographic retention time, the mass spectra and the feasibility of biosynthesis all be considered when proposing a structure for the methyl-branched components. The larvae of these species cannot be distinguished morphologically. They were found to have very little surface lipid, very little hydrocarbons and five to 15 larvae were required to obtain enough surface lipid for one analysis.

The position of the methyl branches has a marked effect on the gas chromatographic retention time (Mold et al., 1966; Nelson, 1978; Kissin et al., 1986; Carlson et al., 1998). The B′ alkanes are dimethylalkanes in which one of the methyl groups is located on carbon 2, 3, or 4 of the carbon chain and were first described in the fire ants, *Solenopsis invicta* and *S. richteri* (Nelson et al., 1980; Thompson et al., 1981). The positions of the two methyl groups on the carbon chain backbone cause the compounds to elute before, after, or with the *n*-alkane of one carbon greater chain length (but one

less number of total carbon atoms), e.g. 30B′ elutes around the 31-carbon *n*-alkane, or stated as: the 32-carbon B′ component elutes approximately the same time as does the 31-carbon *n*-alkane. These compounds, as was the situation herein, are frequently found as minor components of a *n*-alkane peak making them difficult to detect and to identify by either retention time values (Carlson et al., 1998), i.e. equivalent chain length (Miwa, 1963) or Kováts Indices (Kováts, 1965) or mass spectrometry alone.

The mass spectra of methyl-branched alkanes are characterized by the presence of fragment ions formed by carbon–carbon bond cleavage adjacent to the carbon containing the methyl group. The nominal mass of the ion is odd-numbered. However, the fragmentation process of the methyl-branched alkanes also includes the loss of a hydrogen atom resulting in an even-numbered ion of similar or greater intensity than the odd-mass ion. This results in a pair of ions in the mass spectrum which are essential for correct interpretation (Nelson, 1978). The presence of a second methyl group in the fragment ion suppresses the loss of a hydrogen atom so that the fragment remains an odd-numbered mass (McCarthy et al., 1968; Nelson and Sukkestad, 1970). However, if the second methyl group is on carbon 2, there is little suppression of the loss of a hydrogen atom and the even-mass ion is of similar intensity to that of the odd-mass ion (Pomonis et al., 1989; Schulz, 2001). This phenomena was observed in mass spectra of the methyl branched alkanes of these flea beetles with a methyl branch on carbon 2.

The first definitive report of 2,*X*-dimethylalkanes in insects was of mixtures of isomers of dimethyloctacosanes and dimethyltriacontanes in which *X* had values of 6, 8, 10, 12, 14, 16, 18, 20 and 22 in the adult screwworm, *Cochliomyia hominivorax* (Pomonis, 1989). 2,6-Dimethyloctacosane had previously been reported in the Colorado potato beetle (Maliński et al., 1986) and definitive mass spectra were later published (Szafranek et al., 1994).

The adult *Aphthona* beetles had mixtures of B′ isomers similar to those found in the screwworm. The differences in the separation of the methyl branch points caused the 2,12-and 2,10-isomers to elute on the leading edge of the *n*-alkane peak and those of the 2,6- and 2,4-isomers to elute on the tailing edge of the *n*-alkane peak. Thus, the 2,6-isomer eluted just before the 3,7-isomer. Whereas there was a homologous series of 2,6-isomers from 28B′ to 32B′, the only 2,4-isomer detected was 2,4-dimethyloctacosane (28B′). Structures with 3,7-dimethyl branching were found only at 29B′ and 31B′ and those with 2,12- and 2,10-dimethyl branching only at 30B′ and 32B′. The structures for the even carbon-numbered dimethylalkanes were drawn assuming the methyl groups were added early during chain elongation as had been shown for methyl-branched alkanes (Dillwith et al., 1982) and for very long-chain methyl-branched alcohols (Nelson and Fatland, 1992). However, alternate structures can be drawn based on the mass spectra, e.g. 2,6 and 2,22 methyl branching positions on 28B′ gave similar mass spectra (Schulz, 2001).

Those B′ components with an odd number of carbons in the backbone, e.g. 29B′ and 31B′ (Table 2), required a different approach to determining the position of the second methyl branch. Based on our current understanding of methylalkane biosynthesis, the straight-chain tail beyond the last (second) methyl branch point cannot contain an odd number of carbon atoms. Thus, 29B′ and 31B′ were identified as 2,23-dimethylnonacosane and 2,25-dimethylhentriacontane, respectively, with an even number of carbons beyond the last methyl branch point. These structures, also with an even number of carbons between the methyl branch points, could be synthesized beginning with a primer derived from the amino acid leucine. Leucine has been shown to be the primer for the synthesis of 2-methylalkanes with an odd-numbered carbon chain length (odd-numbered carbon backbone) (Blailock et al., 1976; Charlton and Roelofs, 1991).

The first published mass spectra of terminally branched trimethylalkanes were for a homologous series of 3,7,11- and 4,8,12-trimethylalkanes from *Atta colombia* (Martin and MacConnell, 1970). We have now identified 2,10,12-trimethylalkanes in two species of *Aphthona*. Other terminally branched trimethylalkanes (C′), i.e. one of the methyl groups is on carbon 2, 3, or 4 of the carbon chain, have been characterized by GC-MS but in most reports the mass spectra were not published (see discussion in Nelson, 2001). When the structures of methyl-branched alkanes with multiple methyl groups were first described in insects (Martin and MacConnell, 1970; Nelson and Sukkestad, 1970), the number of methylene groups between

carbons containing the methyl groups had been considered to be three or multiples of three. Based on the mechanism of biosynthesis, it was predicted that the number of methylene groups between any two branch points would be an odd number (Nelson, 1993; Nelson and Blomquist, 1995). However, in rare instances, structures with two methylenes between the methyl branch points have been reported: 2,5-dimethylheptadecane in the eastern hemlock looper *Lambdina fiscellaria fiscellaria* (Gries et al., 1991). For a discussion of other reports of 0, 1, or 2 methylenes between methyl branch points, but for which no mass spectra were published, see Nelson, 1978; Blomquist et al., 1987. There is some indication that the presence of two methyl branch points separated by a single methylene results in the formation of an additional ion or pair of ions in the mass spectrum. The even-mass ion of the pair is frequently the largest. This fragmentation is evident in Fig. 3c, Fig. 5a,b and in mass spectra published for: 13,15-dimethylheptacosane in *Solenopsis invicta* (Nelson et al., 1980); 13,15,17-trimethylnonacosane in *Coptotermes formosanus* (Haverty et al., 1996); 13,15,19-trimethylhentriacontane and 13,15,21- trimethyltritriacontane in *Pogonomyrmex barbatus* (Nelson et al., 2001); and 2,18,20-trimethyltetratriacontane, 2,18,20-trimethylhexatriacontane and 2,24,26-trimethyldotetracontane in *Helicoverpa zea* (Nelson, 2001).

The finding of C′ components in which a pair of methyl branch points are separated by a single methylene is rare. Our current understanding of biosynthetic pathways allows for the synthesis of such a structure by the sequential addition of two molecules of propionic acid (as methylmalonyl-CoA) during carbon chain elongation. Thus, the structures presented herein meet the three criteria that a proposed structure must meet in order to be considered valid: (1) supported by the mass spectrum; (2) have an equivalent chain length (or Kováts Index) expected from the proposed structure; and (3) be biosynthetically feasible. The present finding of the B′ isomers, and in particular the C′ components, demonstrates the value of using a mass spectrometer as the detector in gas chromatography.

The results here, as well as in a number of the cited papers, show the large number of structures possible in a hydrocarbon sample and indicate the wide variation in specificity that exists in biosynthetic pathways in insects and other organisms. Mass spectra of relatively short-chain hydrocarbons with methyl groups on adjacent carbons found in cyanobacteria have recently been published (Köster et al., 1999). For reviews and tabulations of occurrences of methyl-branched hydrocarbons see Nelson (1978), Blomquist et al. (1987), Lockey (1988), Nelson (1993) and Nelson and Blomquist (1995). Whether the relatively unique mixtures of methyl-branched hydrocarbons and unsaturated hydrocarbons play a role in the behavior and/or success or failure of these flea beetles to establish at a specific release site remains to be determined.

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